Editorial overview
The importance of being RCR

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Since 1993, the FDA's CBER has issued recommendations and guidelines for the testing of retroviral vector stocks for the presence of replication-competent retrovirus (RCR). These guidelines have evolved over the years and at the end of last month (September 2001) a group of experts from all over the world met in the framework of the 2nd Informal Working Group Meeting on Biological Standardisation in Gene Therapy at the National Institute for Biological Standards and Control (NIBSC) in Potters Bar near London, UK, to discuss methods to ensure the standardization of such RCR tests worldwide.

RCR testing is based on the reasonable fear that such replicating retrovirus might, after multiple rounds of infection and amplification, eventually integrate near or in a cellular gene, disrupting the regulation of its expression and resulting in a triggering of the tumorigenic process. RCR might arise from recombination events between the vector, the packaging constructs used to generate the recombinant virus stocks, and/or endogenous retroviral sequences. It has been shown by us and others that packaging of RNAs lacking a packaging signal occurs at a fairly high frequency, even in the presence of packaging signal carrying vector RNA, thus enabling RNA recombination via template switching during the reverse transcription process within the virion.

Much of our fear of RCR is based upon the fact that oncogenes were originally discovered in animals through their activation by retroviruses integrating in their vicinity. Additionally, one seminal paper is repeatedly cited to underline that such a mechanism can also occur in primates, and, by analogy, in man. In 1992, Donahue and colleagues published the results of a study in which ten primates were each injected with 1×10^9 cells producing replication-competent virus. Three of the primates subsequently went on to develop lymphomas [1].

Interestingly, however, there are more lessons to be learned from this study than immediately meets the eye. Of the ten monkeys treated, only three developed lymphoma, even though all animals had received high-dose total body irradiation. Further, the seven monkeys that were able to control the retrovirus infection had good anti-MLV humoral antibody titres even in the face of the severe immunosuppression induced by the irradiation. The three monkey that did develop lymphoma were analyzed further, and both recombinant retroviruses between the vector and the packaging construct, as well as recombinants between this and endogenous murine retroviruses, could be isolated [2,3]. Interestingly, these were present at a relatively high copy number (between 12 and 25), which is not really consistent with the infection of cells by replication-competent virus which then blocks cellular receptors and prevents further infection resulting in mainly low copy numbers, and possibly pointing to cellular retrotransposition events [4]. Furthermore, our studies on the kinetics of replication of these retroviruses suggest a lower titre of detectable virus than would perhaps be expected for a replication-competent MLV [5]. This could be interpreted to mean that the large number of infections followed by amplification, which are considered necessary to eventually lead to integration near a cellular proto-oncogene and trigger the transformation process, may not have occurred. Indeed, it cannot be excluded that the radiation might have played a role in the tumour etiology, since a causal link between the presence of the recombined retroviruses and the lymphomas has still not been established to date. In fact, this study is the only one that suggests that replication competent MLVs may induce the tumorigenic process in primates. In four further studies, both in immunosuppressed and in normal macaques, no evidence for such a process could be obtained [6,7] [Khan *et al.* 1996 unpublished Gene Therapy Forum].

It is clear that regulators and investigators have to be mindful of results suggesting a possible risk to patients, and indeed to the broader population. To date, most gene therapies have involved either *ex vivo* transduction of cells before their (re)infusion into patients, or the direct application of virus to the patient. In both settings it is feasible, and correct in view of the potential risk, to comprehensively test for all things that may possibly harm the patient, including RCR. But what of current and future protocols in which we will not be able to exclude the possibility of RCR?

In 1998, Long and colleagues published an evaluation of 128 patients receiving intra-cerebral inoculation of retrovirus producing packaging cells [8]. In none of these patients could evidence of RCR be obtained either by PCR or by culture assays even though 41 patients had anti-retroviral antibodies or vector DNA detectable in their peripheral blood leukocytes. In such protocols, in which virus producer cells are directly introduced into patients either in an immuno-privileged site such as the brain, or within some sort of immunoprotective device, it will not be possible to exclude the formation of RCR. Similarly, there is a growing trend towards the use of (conditionally) replication-competent vectors in tumor therapy stemming from a gradual realization that the one-hit kinetics of current replication-incompetent viruses may not be efficient enough to bring therapeutic gene products into enough cancer cells to give a realistic therapeutic effect. Here again the search for RCR would be out of place, since the very vector systems themselves are designed to replicate, albeit to a limited or controlled extent, within the patient.

So what are the real dangers of RCR? It is difficult to tell. The popular interpretation of the Donahue study suggests a 30% risk of rapid induction of lymphoma in a severely immunocompromised patient. But did the lymphomas really arise as a result of the introduction of RCR? The data seem to suggest that even in such severely immunocompromised primates, the majority will be able to mount a highly effective anti-viral humoral response resulting in rapid virus clearance.

Are the macaques studied in the Donahue, Cornetta and Khan experiments good models for man? Replication-competent gibbon ape leukaemia virus is, of course, pathogenic in gibbons. But this virus has adapted itself to the gibbon and uses a different receptor to enter cells. It is probably not a good model for the effects of a MLV vector using a native amphotropic envelope. There is also, of course, a huge population of individuals infected with HIV or HTLV. To date, there has, however, not been a single report of tumorigenicity caused by the classical promoter/enhancer insertion mechanism in this population. HIV causes rapid cell death in its main target CD4+ lymphocyte; however, it also infects a wide variety of other cells, and some of these cells represent a long-lived virus reservoir. HTLV causes a cell proliferation. In these long-term HIV- or HTLV-infected cells the chances are that we would have seen some insertional mutagenesis effects if they represented an important mechanism of tumorigenesis.

So where do we stand? Similarly to xenotransplantation, we should not be frozen into inactivity by perceived risks associated with viruses [5]. Rather, we should go forward and try and amass a body of information in carefully controlled conditions that will let us truly assess the danger of RCR in humans. Most of the information currently available from clinical trials with retroviral vectors has been derived in an RCR-free setting. The way now has to be to go forward and enlarge our understanding of what might or might not happen if RCR is present - but this only makes sense in the context of a therapeutic need. And this will be given in the near future with the increasing requirement for the implantation of retrovirus producing cells, and the use of (conditionally) replicating vectors (see Weber *et al.*, in this issue).

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